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(54) Title: PROTEIN WHICH INTERACTS WITH THE HUNTINGTON'S DISEASE GENE PRODUCT, cDNA CODING THEREFOR, AND ANTIBODIES THERETO		
(57) Abstract <p>A protein, designated as HIP1, interacts differently with the gene product of a normal (16 CAG repeat) and an expanded (> 44 CAG repeat) HD gene. The HIP1 protein originally isolated from the yeast two-hybrid screen is encoded by a 1.2 kb cDNA, devoid of stop codons, that is expressed as a 400 amino acid polypeptide. By further screening of a human frontal cortex cDNA library, and employing the protocol for 5' Rapid Amplification of cDNA ends (RACE), a total of 4795 nucleotides (with an open reading frame of 914 amino acids) of the 10 kb message HIP1 have been isolated to date. Expression of the HIP1 protein was found to be limited to the brain, where the interaction of the HIP1 with the HD protein appears to be necessary for the association of the HD protein with the membrane or specific cytoskeletal components to render it functional. Because HIP1 interacts with expanded HD protein less well than with normal length HD, introduction of additional HIP1 or overexpression of HIP-1 can lead to increased functionality of the defective or normal HD protein. Alternatively, modified forms of the HIP1 which bind more effectively to expanded HD could be introduced to convert the expanded HD protein into a functional molecule.</p>		

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PROTEIN WHICH INTERACTS WITH THE HUNTINGTON'S DISEASE GENE
PRODUCT, cDNA CODING THEREFOR, AND ANTIBODIES THERETO

BACKGROUND OF THE INVENTION

This application relates to a protein designated as HIP1 which interacts with the Huntington's Disease gene product, cDNA coding for HIP1, and methods and compositions relating thereto.

5 "Interacting proteins" are proteins which associate *in vivo* to form specific stable complexes. Non-covalent bonds, including hydrogen bonds, hydrophobic interactions and other molecular associations form between the proteins when two protein surfaces are matched or have affinity for each other. This affinity or match is required for the recognition of the two proteins, and the formation of a stable interaction. Protein-protein interactions are
10 involved in the assembly of enzyme subunits; in antigen-antibody reactions; in forming the supramolecular structures of ribosomes, filaments, and viruses; in transport; and in the interaction of receptors on a cell with growth factors and hormones.

Huntington's disease is an adult onset disorder characterized by selective neuronal loss in discrete regions of the brain and spinal chord that lead to progressive
15 movement disorder, personality change and intellectual decline. From onset, which generally occurs around age 40, the disease progresses with worsening symptoms, ending in death approximately 18 years after onset.

The biochemical cause of Huntington's disease has thus far not been determined. Various theories have been advanced, but each has failed to stand up to
20 experimental evidence designed to test its validity. For example, it was suggested that the selective neuronal loss could be attributed to restricted expression of mRNA or proteins in cells undergoing degeneration. No obviously altered levels of mRNA transcript or protein expression has ever been observed in HD-affected tissues, however.

While the biochemical cause of Huntington's disease has remained elusive, a
25 mutation in a gene within chromosome 4p16.3 subband has been identified and linked to the disease. This gene, referred to as the Huntington's Disease or HD gene, contains three repeat regions, a CAG repeat region and two CCG repeat regions. Testing of Huntington's disease patients has shown that the CAG region is highly polymorphic, and that the number of CAG

repeat units in the CAG repeat region is a very reliable diagnostic indicator of having inherited the gene for Huntington's disease. Thus, in control individuals and in individuals suffering from neuropsychiatric disorders other than Huntington's disease, the number of CAG repeats is between 9 and 35, while in individuals suffering from Huntington's disease the number of CAG repeats is expanded and is 36 or greater.

The protein product encoded by the HD gene has been localized to the cytoplasm, including to the membranes of vesicles on the brain of both normal and HD-affected individuals. To date, no differences have been observed at either the total RNA, mRNA or protein levels between normal and HD-affected individuals. Thus, the function of the HD protein and its role in the pathogenesis of Huntington's Disease remain to be elucidated.

SUMMARY OF THE INVENTION

We have now identified a protein, designated as HIP1, that interacts differently with the gene product of a normal (16 CAG repeat) and an expanded (>44 CAG repeat) HD gene. The HIP1 protein originally isolated from the yeast two-hybrid screen is encoded by a 1.2 kb cDNA, devoid of stop codons, that is expressed as a 400 amino acid polypeptide. By further screening of a human frontal cortex cDNA library, and employing the protocol for 5' Rapid Amplification of cDNA ends (RACE), a total of 4795 nucleotides (with an open reading frame of 914 amino acids) of the 10 kb message HIP1 have been isolated to date. Expression of the HIP1 protein was found to be limited to the brain, where the interaction of the HIP1 with the HD protein appears to be necessary for the association of the HD protein with the membrane or specific cytoskeletal components to render it functional. Because HIP1 interacts with expanded HD protein less well than with normal length HD, introduction of additional HIP1 or overexpression of HIP-1 can lead to increased functionality of the defective or normal HD protein. Alternatively, modified forms of the HIP1 which bind more effectively to expanded HD could be introduced to convert the expanded HD protein into a functional molecule.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 graphically depicts the amount of interaction between HIP1 and Huntingtin proteins with varying lengths of polyglutamine repeat.

DETAILED DESCRIPTION OF THE INVENTION

The HIP1 protein which interacts with the HD gene product was identified using the yeast two-hybrid system described in US Patent No. 5,283,173 which is incorporated herein by reference. Briefly, this system utilizes two chimeric genes or plasmids expressible in a yeast host. The yeast host is selected to contain a detectable marker gene having a binding site for the DNA binding domain of a transcriptional activator. The first chimeric gene or plasmid encodes a DNA-binding domain which recognizes the binding site of the selectable marker gene and a test protein or protein fragment. The second chimeric gene or plasmid encodes for a second test protein and a transcriptional activation domain. The two chimeric genes or plasmids are introduced into the host cell and expressed, and the cells are cultivated. Expression of the detectable marker gene only occurs when the gene product of the first chimeric gene or plasmid binds to the DNA binding domain of the detectable marker gene, and a transcriptional activation domain is brought into sufficient proximity to the DNA-binding domain, an occurrence which is facilitated by protein-protein interactions between the first and second test proteins. By selecting for cells expressing the detectable marker gene, those cells which contain chimeric genes or plasmids for interacting proteins can be identified, and the gene can be recovered and identified.

In testing for Huntington Interacting Proteins, several different plasmids were prepared containing portions of the HD gene. The first four, identified as 16pGBT9, 44pGBT9, 80pGBT9 and 128pGBT9, were GAL4 DNA binding domain-HD in-frame fusions containing nucleotides 314 to 1955 (amino acids 1-540) of the published HD cDNA sequences cloned into the vector pGBT9 (Clontech). These plasmids contain a CAG repeat region of 16, 44, 80 and 128 glutamine-encoding repeats, respectively. A clone (DMK BamHIpGBT9) was made by fusing acDNA encoding the first 544 amino acids of the myotonic dystrophy gene (a gift from R. Komeluk) in-frame with the GAL4-DNA BD of pGBT9 and was used as a negative control.

These plasmids have been used to identify and characterize HIP1, two additional HD-interacting proteins, HIP2 and HIP3 proteins, and can be further used for the identification of additional interacting proteins, and for tests to refine the region on the protein in which the interaction occurs. Thus, a first aspect of the invention is these four plasmids, and the use of this plasmids in identifying HD-interacting proteins. Furthermore, it will be appreciated that the GAL4 DNA-binding and activating domains are not the only domains which can be used in the yeast two-hybrid assay. Thus, in a broader sense, the invention encompasses any chimeric genes or plasmids containing nucleotides 314 to 1955 of the HD gene together with an activating or DNA-binding domain suitable for use in the yeast one, two- or three-hybrid assay for proteins critical in either binding to the HD protein or responsible for regulated expression of the HD gene.

After introducing the plasmids into Y190 yeast host cells, transforming the host cells with an adult human brain MatchmakerTM (Clontech) cDNA library coupled with a GAL4 activating domain, and selecting for the expression of two detectable marker genes to identify clones containing genes for interacting proteins, the activating domain plasmids were recovered and analyzed. As a result of this analysis, three different cDNA fragments were identified as encoding for HD-interacting proteins and designated as HIP1, HIP2 and HIP3. The sequences of HIP1 and HIP3 are given in Seq. ID. Nos 1 and 3. The polypeptides which each encodes are given by Seq. ID Nos. 2 and 4. Further investigation of the HIP1 cDNA resulted in the characterization of an additional region of cDNA totaling 4795 bases and a corresponding protein, the sequences of which are given by Seq ID Nos. 5 and 6, respectively.

The cDNA molecules, particularly those encoding portions of HIP1, can be explored using oligonucleotide probes for example for amplification and sequencing. In addition, oligonucleotide probes complementary to the cDNA can be used as diagnostic probes to localize and quantify the presence of HIP1 DNA. Probes of this type with a one or two base mismatch can also be used in site-directed mutagenesis to introduce variations into the HIP1 sequence which may increase. Thus, a further aspect of the present invention is an oligonucleotide probe, preferably having a length of from 15-40 bases which specifically and selectively hybridizes with the cDNA given by Seq. ID No. 1 or 5 or a sequence complementary thereto. As used herein, the phrase "specifically and selectively hybridizes with" the

cDNA refers to primers which will hybridize with the cDNA under stringent hybridization conditions.

DNA sequencing of the HIP1 cDNA initially isolated from the yeast two-hybrid screen revealed a 1.2 kb cDNA that shows no significant degree of nucleic acid identity with any stretch of DNA using the blastn program at ncbi (blast@ncbi.nlm.nih.gov). When the entire HIP1 cDNA sequence (SEQ ID NO. 5) is translated into a polypeptide, the entire HIP1 cDNA coding (nucleotides 328-3069) is observed to be devoid of stop codons, and to produce a 914 amino acid polypeptide. A polypeptide identity search revealed an identity match over the entire length of the protein (46% conservation) with that of a hypothetical protein from *C. elegans* (ZK370.3 protein; *C. elegans* cosmid ZK370). This *C. elegans* protein shares identity with the mouse talin gene, which encodes a 217 kDa protein implicated with maintaining integrity of the cytoskeleton. It also shares identity with the SLA2/MOP2/ END4 gene from *Saccharomyces cerevisiae*, which is known to code for an essential cytoskeletal associated gene required for the accumulation and or maintenance of plasma membrane H⁺-ATPase on the cell surface. When pairwise comparisons are performed between HIP1 and the *C. elegans* ZK370.3 protein (Genpept accession number celzk370.3), it shows 26% complete identity and an overall 46% level of conservation. Comparative analysis between HIP1 and SLA2/MOP2/ END4 (EMBL accession number Z22811) demonstrate similar conservation (20% identity, 40% conservation).

HIP2 is a 2.0 kb cDNA that encodes all but the 5'-most 33 amino acids of human E2_{25k} ubiquitin conjugating enzyme. The resulting peptide has 100% identity with the previously characterized bovine E2_{25k} protein. The cDNA has 95% nucleotide identity with the bovine cDNA. Ubiquitin-conjugating enzyme is an important component in ubiquitin-mediated protein degradation pathways.

No difference in the strength of the interaction between HIP2 and HD constructs containing either 44 or 15 CAG repeats is detected using a quantitative β -galactosidase assay. The expression pattern of HIP2 (E2_{25k}) in the various parts of the brain and nervous system appears to follow the specific neuropathology observed in HD, although there does not appear to be any difference in expression levels between HD-affected and HD-non-affected individuals.

The third cDNA encoding an HD-interacting protein is a 537 bp cDNA coding for 187 amino acids. A search of known DNA databases did not identify the sequence homology with any known genes. However, when a protein search was performed using the blatsp server, a strong identity between HIP3 and ankyrin-related proteins was observed. The strongest identity was with the D2021.8 gene product of *C. elegans*, an uncharacterized gene, but there is also a 41 % identity with AKR1, a yeast ankyrin repeat-containing protein. Furthermore, when analogous structures with charge conservation over the same amino acid stretch are considered, there is 70 % protein identity. HIP3 also shares approximately 60 % amino acid conservation with human brain specific ankyrins (ankyrin B and ankyrin C). Thus, it is reasonable to conclude that HIP3, like known ankyrins, is a cytoskeletal protein, and may be involved, like previously characterized ankyrins in promoting interactions between the membrane skeleton and other membrane proteins.

Further exploration of these three HD interacting proteins revealed several important facts about HIP1 that implicate it in a significantly in the pathogenesis of Huntington's Disease. First, as shown in Fig. 1, it was found that the strength of the interaction between HD protein and HIP1 is dependent on the number of CAG repeats. Second, it was found that expression of the HIP1 protein is not ubiquitous, but is limited to brain tissue. The highest amounts of expression are in the cortex, with lower levels being seen in the cerebellum, caudate and putamen.

Both HIP1 and HIP3 appear to be proteins which are involved in the maintaining the structural integrity of the cytoskeleton and various components of the cellular membrane, including microtubules and focal adhesions. Based upon this, the HD protein may be associated as part of the cytoskeletal matrix in cells where it is expressed, and our work supports the conclusion that binding of HIP1 to the HD protein is necessary for the functional incorporation of the HD protein into the cell membrane. In this circumstance, the larger polyglutamine tract in huntingtin has a decreased ability for an HIP1-HD interaction. This decreased affinity for each other disrupts the normally strong HD-HIP1-cytoskeletal anchoring association. Further, the HIP1-HD interaction may be a critical interaction at the membranes of synaptic vesicles and a decrease in the affinity of HIP1 for huntingtin may affect protein trafficking or membrane organization throughout

the neuron. Finally, we have demonstrated that HIP1 and HD are both found in the Triton X-100 insoluble membrane compartment of the cell, therefore, a decreased interaction between HIP1 and huntingtin may allow an abnormally subtle amount of huntingtin to be found in subcellular compartments in which it is normally found.

5 As a result of all three of these phenomenon, increased apoptosis can occur in specific neurons within the striatum. This increase in apoptosis arises from an increased susceptibility of polyglutamine-expanded huntingtin to cleavage by apopain, and because more of the expanded forms of the HD protein may be available for cleavage (and subsequent apoptosis) due to the fact they are not as tightly associated at the HD-HIP1-
10 cytoskeletal complex.

 This understanding of a biochemical basis for the pathogenesis of Huntington's Disease opens the doorway to a therapeutic method to ameliorate the pathology in patients expressing huntingtin protein with expanded polyglutamine tracts. In accordance with the method, the patient is treated to increase the amount of HIP1 or an
15 equivalent polypeptide which interacts less well with expanded Huntingtin than with Huntingtin having a CAG repeat region containing 15 to 35 repeats and facilitates the incorporation of Huntingtin into brain cell membranes.

 Increasing expression of HIP1 or an equivalent polypeptide can be accomplished using gene therapy approaches. In general, this will involve introduction of
20 DNA encoding HIP1 in an expressable vector into the brain cells. Vectors which have been shown to be suitable expression systems in mammalian cells include the herpes simplex viral based vectors: pHSV1 (Geller et al. Proc. Natl. Acad. Sci. 87:8950-8954 (1990)); recombinant retroviral vectors: MFG (Jaffee et al. Cancer Res. 53:2221-2226 (1993)); Moloney-based retroviral vectors: LN, LNSX, LNCX, LXSX (Miller and
25 Rosman Biotechniques 7:980-989 (1989)); vaccinia viral vector: MVA (Sutter and Moss Proc. Natl. Acad. Sci. 89:10847-10851 (1992)); recombinant adenovirus vectors : pJM17 (Ali et al Gene Therapy 1:367-384 (1994)), (Berkner K. L. Biotechniques 6:616-624 1988); second generation adenovirus vector: DE1/DE4 adenoviral vectors (Wang and Finer Nature Medicine 2:714-716 (1996)); and Adeno-associated viral vectors:
30 AAV/Neo (Muro-Cacho et al. J. Immunotherapy 11:231-237 (1992)).

Delivery of retroviral vectors to brain and nervous system tissue has been described in US Patents Nos. 4,866,042, 5,082,670 and 5,529,774, which are incorporated herein by references. These patents disclose the use of cerebral grafts or implants as one mechanism for introducing vectors bearing therapeutic gene sequences into the brain, as well as an approach in which the vectors are transmitted across the blood brain barrier.

In addition to increasing the amount of HIP1 present in brain cells of affected individuals, HD lethal phenotype may be rescued by coexpression of a HIP1 and normal sized HD protein within the same cell, specifically within neurons. The over-expression of the normal HD protein and the presence of excess HIP1 in the cell may be able to override the damaging effects of a decreased interaction between HIP1 and an expanded form of the HD protein. Therefore, a "normal state" of interaction of HD with HIP1 will rescue the cell from premature apoptotic death. Thus, a therapeutically desirable mammalian expression vector may include both a region encoding HIP1 and a region encoding normal (less than 35 repeats) HD protein.

To further illustrate the methods of making the materials which are the subject of this invention, and the testing which has established their utility, the following non-limiting experimental procedures are provided.

EXAMPLE 1

IDENTIFICATION OF INTERACTING PROTEINS

GAL4-HD cDNA constructs

An HD cDNA construct (44pGBT9), with 44 CAG repeats was generated encompassing amino acids 1 - 540 of the published HD cDNA. This cDNA fragment was fused in frame to the GAL4 DNA-binding domain (BD) of the yeast two-hybrid vector pGBT9 (Clontech). Other HD cDNA constructs, 16pGBT9, 80pGBT9 and 128pGBT9 were constructed, identical to 44pGBT9 but included only 16, 80 or 128 CAG repeats, respectively.

Another clone (DMKDBamHlpGBT9) containing the first 544 amino acids of the myotonic dystrophy gene (a gift from R. Korneluk) was fused in-frame with the

GAL4-DNA BD of pGBT9 and was used as a negative control. Plasmids expressing the GAL4-BDRAD7 (D. Gietz, unpublished) and SIR3 were used as a positive control for the β -galactosidase filter assay.

The clones IT15-23Q, IT15-44Q and HAP1 were generous gifts from Dr. C. Ross. These clones represent a previously isolated huntingtin interacting protein that has a higher affinity for the expanded form of the HD protein.

Yeast strains, transformations and β -galactosidase assays

The yeast strain Y190 (MATa leu2-3,112, ura3-52, trp1-901, his3- Δ 200, ade2-101, gal4 Δ gal80 Δ , URA3::GAL-lacZ, LYS2::GAL-HIS3,cyc⁺) was used for all transformations and assays. Yeast transformations were performed using a modified lithium acetate transformation protocol and grown at 30 C using appropriate synthetic complete (SC) dropout media.

The β -galactosidase chromogenic filter assays were performed by transferring the yeast colonies onto Whatman filters. The yeast cells were lysed by submerging the filters in liquid nitrogen for 15-20 seconds. Filters were allowed to dry at room temperature for at least five minutes and placed onto filter paper presoaked in Z-buffer (100 mM sodium phosphate (pH7.0) 10 mM KCl, 1 mM MgSO₄) supplemented with 50 mM 2-mercaptoethanol and 0.07 mg/ml 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal). Filters were placed at 37 C for up to 8 hours.

Yeast two-hybrid screening for huntingtin interacting protein (HIP)

cDNAs from an human adult brain MatchmakerTM cDNA library (Clontech) was transformed into the yeast strain Y190 already harboring the 44pGBT9 construct. The transformants were plated onto one hundred 150 mm x 15 mm circular culture dishes containing SC media deficient in Trp, Leu and His. The herbicide 3-amino-triazole (3-AT) (25mM) was utilized to limit the number of false His⁺ positives (31). The yeast transformants were placed at 30 C for 5 days and β -galactosidase filter assays were performed on all colonies found after this time, as described above, to identify β -galactosidase⁺ clones. Primary His⁺/ β -galactosidase⁺ clones were then orderly patched onto a grid on SC

-Trp/-Leu/-His (25 mM 3AT) plates and assayed again for His⁺ growth and the ability to turn blue with a filter assay. Secondary positives were identified for further analysis. Proteins encoded by positive cDNAs were designated as HIPs (Huntingtin Interactive Proteins).

Approximately 4.0×10^7 Trp/Leu auxotrophic transformants were screened and of 14 clones isolated 12 represented the same cDNA (HIP1), and the other 2 cDNAs, HIP2 and HIP3 were each represented only once.

The HIP cDNA plasmids were isolated by growing the His⁺/β-galactosidase⁺ colony in SC -Leu media overnight, lysing the cells with acid-washed glass beads and electroporating the bacterial strain, KC8 (leuB auxotrophic) with the yeast lysate. The KC8 ampicillin resistant colonies were replica plated onto M9 (-Leu) plates. The plasmid DNA from M9⁺ colonies was transformed into DH5-a for further manipulation.

EXAMPLE 2

CONFIRMATION OF INTERACTIONS

The HIP1-GAL4-AD cDNA activated both the lac-Z and His reporter genes in the yeast strain Y190 only when co-transformed with the GAL4-BD-HD construct, but not the negative controls (Figure 1) of the vector alone or a random fusion protein of the myotonin kinase gene. In order to assess the influence of the polyglutamine tract on the interaction between HIP1 and HD, semi-quantitative β-galactosidase assays were performed. GAL4-BD-HD fusion proteins with 16, 44, 80 and 128 glutamine repeats were assayed for their strength of interaction with the GAL4-AD-HIP1 fusion protein.

Liquid β-galactosidase assays were performed by inoculating a single yeast colony into appropriate synthetic complete (SC) dropout media and grown to OD₆₀₀ 0.6-1.5. Five millilitres of overnight culture was pelleted and washed once with 1 ml of Z-Buffer, then resuspended in 100 ml Z-Buffer supplemented with 38 mM 2-mercapto-ethanol, and 0.05% SDS. Acid washed glass beads (~100 ml) were added to each sample and vortexed for four minutes, by repeatedly alternating a 30 seconds vortex, with 30 seconds on ice. Each sample was pelleted and 10 ml of lysate was added to 500 ml of lysis buffer. The samples were incubated in a 30 C waterbath for 30 seconds and then 100 ml of a 4 mg/ml o-nitrophenyl b-D galactopyranoside (ONPG) solution was added to each tube.

The reaction was allowed to continue for 20 minutes at 30 C and stopped by the addition of 500 ml of 1 M Na₂CO₃ and placing the samples on ice. Subsequently, OD420 was taken in order to calculate the β -galactosidase activity with the equation $1000 \times \text{OD420} / (t \times V \times \text{OD600})$ where t is the elapsed time (minutes) and V is the amount of lysate used.

The specificity of the HIP1-HD interaction can be observed using the chromogenic filter assay. Only yeast cells harboring HIP1 and HD activate both the HIS and lacZ reporter genes in the Y190 yeast host. The cells that contain the HIP1 with HD constructs with 80 or 128 CAG repeats turn blue approximately 45 minutes after the cells with the smaller sized repeats (16 or 44).

No difference in the β -galactosidase activity was observed between the 16 and 44 repeats or between the 80 and 128 repeats. However, a significant difference ($p < 0.05$) in activity is seen between the smaller repeats (16 and 44) and the larger repeats (80 and 128). (Figure 1)

EXAMPLE 3

DNA SEQUENCING, cDNA ISOLATION AND 5' RACE

Oligonucleotide primers were synthesized on an ABI PCR-mate oligo-synthesizer. DNA sequencing was performed using an ABI 373 fluorescent automated DNA sequencer. The HIP cDNAs were confirmed to be in-frame with the GAL4-AD by sequencing across the AD-HIP1 cloning junction using an AD oligonucleotide (5'GAA GAT ACC CCA CCA AAC3').

Subsequently, primer walking was used to determine the remaining sequences. A human frontal cortex >4.0 kb cDNA library (a gift from S. Montal) was screened to isolate the full length HIP1 gene. Fifty nanograms of a 558 base pair Eco RI fragment from the original HIP1 cDNA was radioactively labeled with [$\alpha^{32}\text{P}$]-dCTP using nick-translation and the probe allowed to hybridized to filters containing > 105 pfu/ml of the cDNA library overnight at 65 C in Church buffer (see Northern blot protocol). The filters were washed at 65 C for 10 minutes with 1 X SSPE, 15 minutes at 65 C with 1 X SSPE and 0.1 % SDS, then for thirty minutes and fifteen minutes with 1 X SSPE and 0.1 % SDS. The filters were exposed to X-ray film (Kodak, XAR5) overnight at -70 C. Primary

positives were isolated and replated and subsequent secondary positives were hybridized and washed as for the primary screen. The resulting positive phage were converted into plasmid DNA by conventional methods (Stratagene) and the cDNA isolated and sequenced.

In order to obtain the most 5' sequence of the HIP1 gene, a Rapid
5 Amplification of cDNA Ends (RACE) protocol was performed according to the
manufacturers recommendations (BRL). First strand cDNA was synthesized using the
oligo HIP1-242R (5' GCT TGA CAG TGT AGT CAT AAA GGT GGC TGC AGT CC
3'). After dCTP tailing the cDNA with terminal deoxy transferase, two rounds of 35
cycles (94 C 1 minute; 53 C 1 minute; 72 C 2 minutes) of PCR using HIP1-R2 (5' GGA
10 CAT GTC CAG GGA GTT GAA TAC 3') and an anchor primer (5' (CUA)₄ GGC CAC
GCG TCG ACT AGT ACG GGI IGG GII GGG IIG3') (BRL) were performed. The
subsequent 650 base pair PCR product was cloned using the TA cloning system
(Invitrogen) and sequenced using T3 and T7 primers. Sequences ID Nos. 1 and 5 show the
sequence of the HIP1 cDNAs obtained.

EXAMPLE 4

DNA AND AMINO ACID ANALYSES

Overlapping DNA sequence was assembled using the program MacVector
and sent via email or Netscape to the BLAST server at NIH (<http://www.ncbi.nlm.nih.gov>)
20 to search for sequence similarities with known DNA (blastn) or protein (tblastn) sequences.
Amino acid alignments were performed with the program Clustalw.

EXAMPLE 5

FISH DETECTION SYSTEM AND IMAGE ANALYSIS

25 The HIP1 cDNA isolated from the two-hybrid screen was mapped by
fluorescent in situ hybridization (FISH) to normal human lymphocyte chromosomes
counterstained with propidium iodide and DAPI. Biotinylated probe was detected with
avidin-fluorescein isothiocyanate (FITC). Images of metaphase preparations were captured
by a thermoelectrically cooled charge coupled camera (Photometrics). Separate images of
30 DAPI banded chromosomes and FITC targeted chromosomes were obtained. Hybridization

signals were acquired and merged using image analysis software and pseudo colored blue (DAPI) and yellow (FITC) as described and overlaid electronically. This study showed that HIP1 maps to a single genomic locus at 7q11.2.

EXAMPLE 6

NORTHERN BLOT ANALYSIS

RNA was isolated using the single step method of homogenization in guanidinium isothiocyanate and fractionated on a 1.0% agarose gel containing 0.6 M formaldehyde. The RNA was transferred to a hybond N -membrane (Amersham) and crosslinked with ultraviolet radiation.

Hybridization of the Northern blot with b-actin as an internal control probe provided confirmation that the RNA was intact and had transferred. The 1.2 kb HIP1 cDNA was labeled using nick translation and incorporation of $\alpha^{32}\text{P}$ -dCTP. Hybridization of the original 1.2 kb HIP1 cDNA was carried out in Church buffer (0.5 M sodium phosphate buffer, pH 7.2, 2.7% sodium dodecyl sulphate, 1 mM EDTA) at 55 C overnight. Following hybridization, Northern blots were washed once for 10 minutes in 2.0 X SSPE, 0.1% SDS at room temperature and twice for 10 minutes in 0.15 X SSPE, 0.1% SDS. Autoradiography was carried out from one to three days using Hyperfilm (Amersham) film at -70 C.

Analysis of the levels of RNA levels of HIP1 by Northern blot data revealed that the 10 kilo base HIP1 message is present in all tissue assessed. However, the levels of RNA are not uniform, with brain having highest levels of expression and peripheral tissues having less message. No apparent differences in RNA expression was noted between control samples and HD affected individuals.

EXAMPLE 7

TISSUE LOCALIZATION OF HIP1

Tissue localization of HIP1 was studied using a variety of techniques as described below. Subcellular distribution of HIP-1 protein in adult human and mouse brain Biochemical fractionation studies revealed the HIP1 protein was found to be a

membrane-associated protein. No immunoreactivity was seen by Western blotting in cytosolic fractions, using the anti-HIP1-pep1 polyclonal antibody. HIP1 immunoreactivity was observed in all membrane fractions including nuclei (P1), mitochondria and synaptosomes (P2), microsomes and plasma membranes (P3). The P3 fraction contained the most HIP1 compared to other membrane fractions. HIP1 could be removed from membranes by high salt (0.5M NaCl) buffers indicating it is not an integral membrane protein, however, since low salt (0.1- 0.25M NaCl) was only able to partially remove HIP1 from membranes, its membrane association is relatively strong. The extraction of P3 membranes with the non-ionic detergent, Triton X-100 revealed HIP1 to be a Triton X-100 insoluble protein. This characteristic is shared by many cytoskeletal and cytoskeletal-associated membrane proteins including actin, which was used as a control in this study. The biochemical characteristics of HIP1 described were found to be identical in mouse and human brain and was the same for both forms of the protein (both bands of the HIP1 doublet). HIP1 co-localized with huntingtin in the P2 and P3 membrane fractions, including the high-salt membrane extractions, as well as in the Triton X-100 insoluble residue. The subcellular distribution of HIP1 was unaffected by the expression of polyglutamine-expanded huntingtin in transgenic mice and HD patient brain samples.

The localization of HIP1 protein was further investigated by immunohistochemistry in normal adult mouse brain tissue. Immunoreactivity was seen in a patchy, reticular pattern in the cytoplasm, appeared excluded from the nucleus and stained most intensely in a discontinuous pattern at the membrane. These results are consistent with the association of HIP1 with the cytoskeletal matrix and further indicate an enrichment of HIP1 at plasma membranes. Immunoreactivity occurred in all regions of the brain, including cortex, striatum, cerebellum and brainstem, but appeared most strongly in neurons and especially in cortical neurons. As described previously, huntingtin immunoreactivity was seen exclusively and uniformly in the cytosol.

The in situ hybridization studies showed HIP1 mRNA to be ubiquitously and generally expressed throughout the brain. This data is consistent with the immunohistochemical results and was identical to the distribution pattern of huntingtin mRNA in transgenic mouse brains expressing full-length human huntingtin.

Protein Preparation And Western Blotting For Expression Studies

Frozen human tissues were homogenized using a Polytron in a buffer containing 0.25M sucrose, 20mM Tris-HCl (pH 7.5), 10mM EGTA, 2mM EDTA supplemented with 10ug/ml of leupeptin, soybean trypsin inhibitor and 1mM PMSF, then centrifuged at 4,000rpm for 10' at 4 C to remove cellular debris. 100-150ug/lane of protein was separated on 8% SDS-PAGE mini-gels and then transferred to PVDF membranes. Huntingtin and HIP1 were electroblotted overnight in Towbin's transfer buffer (25 mM Tris-HCl, 0.192M glycine, pH8.3, 10% methanol) at 30V onto PVDF membranes (Immobilon-P, Millipore) as described (Towbin et al, *Proc. Nat'l Acad. Sci.(USA)* 76: 4350-4354 (1979)). Membranes were blocked for 1 hour at room temperature in 5% skim milk/ TBS (10mM Tris-HCl, 0.15M NaCl, pH7.5). Antibodies against huntingtin (pAb BKP1, 1:500), actin (mAb A-4700, Sigma, 1:500) or HIP1 (pAb HIP-pep1, 1:200) were added to blocking solution for 1 hour at room temperature. After 3 x 10 minutes washes in TBS-T (0.05% Tween-20/TBS), secondary Ab (horseradish peroxidase conjugated IgG, Biorad) was applied in blocking solution for 1 hour at room temperature. Membranes were washed and then incubated in chemiluminescent ECL solution and visualized using Hyperfilm-ECL film (Amersham).

Generation of Antibodies

The generation of huntingtin specific antibodies GHM1 and BKP1 is described elsewhere (Kalchman, et al., *J. Biol. Chem.* 271: 19385-19394 (1996)). The HIP1 peptide (VLEKDDLMDMDASQQN, a.a. 76-91 of Seq. ID No. 2) was synthesized with Cys on the N-terminus for the coupling, and coupled to Keyhole limpet hemocyanin (KLH) (Pierce) with succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Pierce). Female New Zealand White rabbits were injected with HIP1 peptide-KLH and Freund's adjuvant. Antibodies against the HIP1 peptide were purified from rabbit sera using affinity column with low pH elution. Affinity column was made by incubation of HIP1 peptide with activated thio-Sepharose (Pharmacia).

Western blotting of various peripheral and brain tissues were consistent with the RNA data. The HIP1 protein levels observed was not ubiquitous. The protein

expression is limited to brain tissue, with highest amounts seen in the cortex and lower levels seen in the cerebellum and caudate and putamen.

More regio-specific analysis of HIP1 expression in the brain revealed no differential expression pattern in affected individuals when compared to normal controls, with highest levels of expression seen in both controls and HD patients in the cortical regions.

EXAMPLE 8

CO-IMMUNOPRECIPITATION OF HIP1 WITH HUNTINGTIN

Confirmation of the HD-HIP1 interaction was performed using coimmunoprecipitation as follows. Control human brain (frontal cortex) lysate was prepared in the same manner as for subcellular localization study. Prior to immunoprecipitation, tissue lysate was centrifuged at 5000 rpm for 2 minutes at 4 C, then the supernatant was pre-cleared by the incubated with excess amount of Protein A-Sepharose for 30 minutes at 4 C, and centrifuged at the same condition. Fifty microlitres of supernatant (500 mg protein) was incubated with or without antibodies (10 ug of anti-huntingtin GHM1 (Kalchman, et al. 1996) or anti-synaptobrevin antibody) in the total 500 ul of incubation buffer (20mM Tris-Cl (pH7.5), 40mM NaCl, 1mM MgCl₂) for 1 hour at 4 C. Twenty microlitres of Protein A-Sepharose (1:1 suspension, for GHM1 and no antibody control) or Protein G-Sepharose (for anti-synaptobrevin antibody; Pharmacia) was added and incubated for 1 hour at 4 C. The beads were washed with washing buffer (incubation buffer containing 0.5 % Triton X-100) three times. The samples on the beads were separated using SDS-PAGE (7.5% acrylamide) and transferred to PVDF membrane (Immobilon-P, Millipore). The membrane was cut at about 150 kDa after transfer for Western blotting (as described above). The upper piece was probed with anti-huntingtin BKP1 (1/1000) and lower piece with anti-HIP1 antibody (1/300).

The results showed that when an anti-HIP1 polyclonal antibody was immunoreacted against a blot containing the GHM1 immunoprecipitates from the brain lysate a doublet was observed at approximately 100 kDa was. When GHM1 was immunoreacted against the same immunoprecipitate the 350 kDa HD protein was also seen. The

specificity of the HD-HIP1 interaction is seen as no immunoreactive bands seen are as a result of the proteins adsorbing to the Protein-A-Sepharose (Lysate + No Antibody) or when a random, non related antibody (Lysate + anti-Synaptobrevin) is used as the immunoprecipitating antibody.

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EXAMPLE 9

Subcellular fractionation of brain tissue

Cortical tissue (20-100 mg/ml) was homogenized, on ice, in a 2 ml pyrex-teflon IKA-RW15 homogenizer (Tekmar Company) in a buffer containing 0.303M sucrose, 20mM Tris-HCl pH 6.9, 1mM $MgCl_2$, 0.5mM EDTA, 1mM PMSF, 1mM leupeptin, soybean trypsin inhibitor and 1mM benzamidine (Wood et al., *Human Molec. Genet.* 5: 481-487 (1996)).

Crude membrane vesicles were isolated by two cycles of a three-step differential centrifugation protocol in a Beckman TLA 120.2 rotor at 4 C based on the methods of Wood et al (1996). The first step precipitated cellular debris and nuclei from tissue homogenates for 5 minutes at 1300 x g (P1). The 1300 x g supernatant was subsequently centrifuged for 20 minutes at 14 000 x g to isolate synaptosomes and mitochondria (P2). Finally, microsomal and plasma membrane vesicles were collected by a 35 minute centrifugation at 142 000 x g (P3). The remaining supernatant was defined as the cytosolic fraction.

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High salt extraction of membranes

Aliquots of P3 membranes were twice suspended at 2mg/ ml in 0.5M NaCl, 10mM Tris-HCl, 2mM $MgCl_2$, pH7.2, containing protease inhibitors (see above). The same buffer without NaCl was used as a control. The membrane suspensions were incubated on ice for 30 minutes and then centrifuged at 142 000 x g for 30 minutes.

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Extraction of cytoskeletal and cytoskeletal-associated proteins.

To extract cytoskeletal proteins, crude membrane vesicles from the P3 fraction membrane were suspended in a volume of Triton X-100 extraction buffer to give a

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protein: detergent ratio of 5:1. The composition of the Triton X-100 extraction buffer was based on the methods of Arai et al., *J. Neuroscience* 38: 348-357 (1994) and contained 2% Triton X-100, 10mM Tris-HCl, 2mM MgCl₂, 1mM leupeptin, soybean trypsin inhibitor, PMSF and benzamidine. Membrane pellets were suspended by hand with a round-bottom teflon pestle, and placed on ice for 40 minutes. Insoluble cytoskeletal matrices were precipitated for 35 minutes at 142 000 x g in a Beckman TLA 120.2 rotor. The supernatant was defined as non-cytoskeletal-associated membrane or membrane-associated protein and was removed. The remaining pellet was extracted with Triton X-100 a second time using the same conditions. We defined the final pellet as cytoskeletal and cytoskeletal-associated protein.

Solubilization of protein and analysis by SDS-PAGE and Western Blotting

Membrane and cytoskeletal protein was solubilized in a minimum volume of 1% SDS, 3M urea, 0.1mM dithiothreitol in TBS buffer and sonicated. Protein concentration was determined using the BioRad DC Protein assay and samples were diluted at least 1 X with 5 X sample buffer (250mM Tris-HCl pH 6.8, 10% SDS, 25% glycerol, 0.02% bromophenol blue and 7% 2-mercaptoethanol) and were loaded on 7.5% SDS-PAGE gels (Bio-Rad Mini-PROTEIN II Cell system) without boiling. Western blotting was performed as described above.

Immunohistochemistry

Brain tissue was obtained from a normal C57BL/6 adult (6 months old) male mouse sacrificed with chloroform then perfusion-fixed with 4% v/v paraformaldehyde/0.01 M phosphate buffer (4% PFA). The brain tissues were removed, immersion fixed in 4% PFA for 1 day, washed in 0.01M phosphate buffered saline, pH 7.2 (PBS) for 2 days, and then equilibrated in 25% w/v sucrose PBS for 1 week. The samples were then snap-frozen in Tissue Tek molds by isopentane cooled in liquid nitrogen. After warming to -20 C, frozen blocks derived from frontal cortex, caudate/putamen, cerebellum and brainstem were cut into 14 mm sections for immunohistochemistry. Following washing in PBS, the tissue sections were blocked using 2.5% v/v normal goat serum for 1 hour at room

temperature. Primary antibodies diluted with PBS were applied to sections overnight at 4 C. Optimal dilutions for the polyclonal antibodies BKP1 and HIP1 were 1:50. Using washes of 3 x 5 minutes in PBS at room temperature, sections were sequentially incubated with biotinylated secondary antibody and then an avidin-biotin complex reagent (Vecta Stain ABC Kit, Vector) for 60 minutes each at room temperature. Color was developed using 3-3'-diaminobenzidine tetrahydrochloride and ammonium nickel sulfate.

For controls, sections were treated as described above except that HIP1 antibody aliquots were preabsorbed with an excess of HIP1 peptide as well as a peptide unrelated to HIP1 prior to incubation with the tissue sections.

In situ hybridization

In situ hybridization was performed as previously described with some modification (Suzuki et al, *BBRC* 219: 708-713 (1996)). The RNA probes were prepared using the plasmid gt149 (Lin, B., et al., *Human Molec. Genet.* 2: 1541-1545 (1994)) or a 558 subclone of HIP1. The anti-sense and sense single-stranded RNA probes were synthesized using T3 and T7 RNA polymerases and the In Vitro Transcription Kit (Clontech) with the addition of [α^{35} S]-CTP (Amersham) to the reaction mixture. Sense RNA probes were used as negative controls. For HIP1 studies normal C57BL/6 mice were used. Huntingtin probes were tested on two different transgenic mouse strains expressing full-length huntingtin, cDNA HD10366(44CAG) C57BL/6 mice and YAC HD10366(18CAG) FVB/N mice. Frozen brain sections (10um thick) were placed onto silane-coated slides under RNase-free conditions. The hybridization solution contained 40% w/v formamide, 0.02M Tris-HCl (pH 8.0), 0.005M EDTA, 0.3 M NaCl, 0.01M sodium phosphate (pH 7.0), 1x Denhardt's solution, 10% w/v dextran sulfate (pH 7.0), 0.2% w/v sarcosyl, yeast tRNA (500mg/ml) and salmon sperm DNA (200mg/ml). The radiolabelled RNA probe was added to the hybridization solution to give 1 x 10⁶ cpm/200 ul/ section. Sections were covered with hybridization solution and incubated on formamide paper at 65 C for 18 hours. After hybridization, the slides were washed for 30 minutes sequentially with 2x SSC, 1x SSC and high stringency wash solution (50% formamide, 2x SSC and 0.1M dithiothreitol) at 65 C, followed by treatment with Rnase A

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(1mg/ml) at 37 C for 30 minutes, then washed again and air-dried. The slides were first exposed on autoradiographic film (b-max, Amersham, UK) for 48 hours and developed for 4 minutes in Kodak D-19 followed by a 5 minute fixation in Fuji-fix. For longer exposures, the slides were dipped in autoradiographic emulsion (50% w/v in distilled water, NR-2, Konica, Japan), air-dried and exposed for 20 days at 4 C then developed as described. Sections were counterstained with methyl green or Giemsa solutions.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kalchman, Michael

Goldberg, Paul

Hayden, Michael R.

(ii) TITLE OF INVENTION: Protein Which Interacts with the Huntington's Disease Gene Product, cDNA Coding Therefor, and Antibodies Thereto

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Oppedahl & Larson

(B) STREET: 1992 Commerce Street Suite 309

(C) CITY: Yorktown

(D) STATE: NY

(E) COUNTRY: USA

(F) ZIP: 10598

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 Kb storage

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: MS DOS 5.0

(D) SOFTWARE: WordPerfect

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Larson, Marina T.

(B) REGISTRATION NUMBER: 32038

(C) REFERENCE/DOCKET NUMBER: UBC.P-013

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (914) 245-3252

(B) TELEFAX: (914) 962-4330

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1164

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(ix) FEATURE: cDNA for Huntingtin-interacting protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

- 22 -

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 386

(B) TYPE: protein

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: no

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(ix) FEATURE: Huntingtin-interacting protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Ala	Glu	Gln	Gln	His 170	Leu	Arg	Gln	Gln	Ala 175	Ala	Asp	Asp	Cys	Glu 180
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Glu	Lys	Ala	Gln	Arg 200	Ser	Leu	Ser	Glu	Ile 205	Glu	Arg	Lys	Ala	Gln 210
Ala	Asn	Glu	Gln	Arg 215	Tyr	Ser	Lys	Leu	Lys 220	Glu	Lys	Tyr	Ser	Glu 225
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Arg	Glu	Lys	Lys	Glu 260	Leu	Glu	Asp	Ser	Leu 265	Glu	Arg	Ile	Ser	Asp 270
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Leu	Lys	Gln	Glu	Leu 290	Gly	Thr	Ser	Gln	Arg 295	Glu	Leu	Gln	Val	Leu 300
Gln	Gly	Ser	Leu	Glu 305	Thr	Ser	Ala	Gln	Ser 310	Glu	Ala	Asn	Trp	Ala 315
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(xi)SEQUENCE DESCRIPTION: SEQ ID NO:3:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

- 25 -

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Glu	Ala	Gly	Tyr	Asp	Val	Arg	Gln	Pro	Asp	Lys	Glu	Asn	Val	Thr	
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Leu	Leu	His	Trp	Ala	Ala	Ile	Asn	Asn	Arg	Ile	Asp	Leu	Val	Lys	
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Tyr	Tyr	Ile	Ser	Lys	Gly	Ala	Ile	Val	Asp	Gln	Leu	Gly	Gly	Asp	
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Leu	Asn	Ser	Thr	Pro	Leu	His	Trp	Asp	Thr	Arg	Gln	Gly	His	Leu	
				110					115					120	
Ser	Met	Val	Val	Gln	Leu	Met	Lys	Tyr	Gly	Ala	Asp	Pro	Ser	Leu	
				125					130					135	
Ile	Asp	Gly	Glu	Gly	Cys	Ser	Cys	Ile	His	Leu	Ala	Ala	Gln	Phe	
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4846

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(ix) FEATURE: cDNA for Huntingtin-interacting protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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- 26 -

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TCCTGCGAGC	CTCAGCCCTG	TCAGAACATA	TCAGCCCTGT	GGTGGTGATC	900
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CCTCATGGAC	ATGGATGCCT	CTCAGCAGAA	TTTATTTGAC	AACAAGTTTG	1000
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CCTGGTGGAC	AAGGAGATGG	CGGCCACTTC	AGCTGCTATT	GAAACTTGCA	2350
CGGCCAGAAT	AGAGGAGATG	CTCAGCAAAT	CCCGAGCAGG	AGACACAGGA	2400
GTCAAATTG3	AGGTGAATGA	AAGGATCCTT	CGTTGCTGTA	CCAGCCTCAT	2450
GCAAGCTATT	CAGGTGCTCA	TCGTGGCCTC	TAAGGACCTC	CAGAGAGAGA	2500
TTGTGGAGAG	CGGCAGGGGT	ACAGCATCCC	CTAAAGAGTT	TTATGCCAAG	2550
AACTCTCGAT	GGACAGAAGG	ACTTATCTCA	GCCTCCAAGG	CTGTGGGCTG	2600
GGGAGCCACT	GTCATGGTGG	ATGCAGCTGA	TCTGGTGGTA	CAAGGCAGAG	2650
GGAAATTTGA	GGAGCTAATG	GTGTGTTCTC	ATGAAATTGC	TGCTAGCACA	2700
GCCCAGCTTG	TGGCTGCATC	CAAGGTGAAA	GCTGATAAGG	ACAGCCCCAA	2750
CCTAGCCCAG	CTGCAGCAGG	CCTCTCGGGG	AGTGAACCAG	GCCACTGCCG	2800
GCGTTGTGGC	CTCAACCATT	TCCGGCAAAT	CACAGATCGA	AGAGACAGAC	2850
AACATGGACT	TCTCAAGCAT	GACGCTGACA	CAGATCAAAC	GCCAAGAGAT	2900

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GGATTCTCAG	GTTAGGGTGC	TAGAGCTAGA	AAATGAATTG	CAGAAGGAGC	2950
GTCAAAAAC	GGGAGAGCTT	CGGAAAAAGC	ACTACGAGCT	TGCTGGTGTT	3000
GCTGAGGGCT	GGGAAGAAGG	AACAGAGGCA	TCTCCACCTA	CACTGCAAGA	3050
AGTGGTAACC	GAAAAAGAAT	AGAGCCAAAC	CAACACCCCA	TATGTCAGTG	3100
TAAATCCTTG	TTACCTATCT	CGTGTGTGTT	ATTTCCCCAG	CCACAGGCCA	3150
AATCCTTGGA	GTCCAGGGG	CAGCCACACC	ACTGCCATTA	CCCAGTGCCG	3200
AGGACATGCA	TGACACTTCC	CAAAGATCCC	TCCATAGCGA	CACCCTTTCT	3250
GTTTGACCC	ATGGTCATCT	CTGTTCTTTT	CCCGCCTCCC	TAGTTAGCAT	3300
CCAGGCTGGC	CAGTGCTGCC	CATGAGCAAG	CCTAGGTACG	AAGAGGGGTG	3350
GTGGGGGGCA	GGGCCACTCA	ACAGAGAGGA	CCAACATCCA	GTCCTGCTGA	3400
CTATTTGACC	CCCACAACAA	TGGGTATCCT	TAATAGAGGA	GCTGCTTGTT	3450
GTTTGTTGAC	AGCTTGGAAG	GGGAAGATCT	TATGCCTTTT	CTTTTCTGTT	3500
TTCTTCTCAG	TCTTTTTCAGT	TTCATCATTT	GCACAAACTT	GTGAGCATCA	3550
GAGGGCTGAT	GGATTCCAAA	CCAGGACACT	ACCCTGAGAT	CTGCACAGTC	3600
AGAAGGACGG	CAGGAGTGTC	CTGGCTGTGA	ATGCCAAAGC	CATTCTCCCC	3650
CTCTTTGGGC	AGTGCCATGG	ATTTCCACTG	CTTCTTATGG	TGGTTGGTTG	3700
GGTTTTTTTG	TTTTGTFTTT	TTTTTTTAAAG	TTTCACTCAC	ATAGCCAACT	3750
CTCCCAAAGG	GCACACCCCT	GGGGCTGAGT	CTCCAGGGCC	CCCCAACTGT	3800
GGTAGCTCCA	GCGATGGTGC	TGCCCAGGCC	TCTCGGTGCT	CCATCTCCGC	3850
CTCCCACTG	ACCAAGTGCT	GGCCCCACCA	GTCCATGCTC	CAGGGTCAGG	3900
CGGAGCTGCT	GAGTGACAGC	TTTCCTCAAA	AAGCAGAAGG	AGAGTGAGTG	4000
CCTTTCCCTC	CTAAAGCTGA	ATCCCGCGG	AAAGCCTCTG	TCCGCCTTTA	4050
CAAGGGAGAA	GACAACAGAA	AGAGGGACAA	GAGGGTTCAC	ACAGCCCAGT	4100
TCCCGTGACG	AGGCTCAAAA	ACTTGATCAC	ATGCTTGAAT	GGAGCTGGTG	4150
AGATCAACAA	CACTACTTCC	CTGCCGGAAT	GAAGTGTCCG	TGAATGGTCT	4200
CTGTCAAGCG	GGCCGTCTCC	CTTGCCCCAG	AGACGGAGTG	TGGGAGTGAT	4250
TCCCAACTCC	TTTCTGCAGA	CGTCTGCCTT	GGCATCCTCT	TGAATAGGAA	4300
GATCGTTCCA	CTTTCTACGC	AATTGACAAA	CCCGGAAGAT	CAGATGCAAT	4350
TGCTCCCATC	AGGGAAGAAG	CCTATACTTG	GTTTGCTACC	CTTAGTATTT	4400
ATTACTAACC	TCCCTTAAGC	AGCAACAGCC	TACAAAGAGA	TGCTTGGAGC	4450
AATCAGAACT	TCAGGTGTGA	CTCTAGCAAA	GCTCATCTTT	CTGCCCCGCT	4500
ACATCAGCCT	TCAAGAATCA	GAAGAAAGCC	AAGGTGCTGG	ACTGTTACTG	4550
ACTTGATCC	CAAAGCAAGG	AGATCATTTG	GAGCTCTTGG	GTCAGAGAAA	4600
ATGAGAAAGG	ACAGAGCCAG	CGGCTCCAAC	TCCTTTTCAGC	CACATGCCCC	4650
AGGCTCTCGG	TGCCCTGTGG	ACAGGATGAG	GACAGAGGGC	ACATGAACAG	4700
CTTGCCAGGG	ATGGGCAGCC	CAACAGCACT	TTTCCTCTTC	TAGATGGACC	4750
CCAGCATTTA	AGTGACCTTC	TGATCTTGGG	AAAACAGCGT	CTTCCTTCTT	4800
TATCTATAGC	AACTCATTGG	TGGTAGCCAT	CAAGCACTTC	GGAATT	4846

(2) INFORMATION FOR SEQ ID NO:6

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 924

(B) TYPE: protein

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: no

(vi) ORIGINAL SOURCE:

(A) ORGANISM: human

(ix) FEATURE: Huntingtin-interacting protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Met	Ser	Arg	Met	Trp	Gly	His	Leu	Ser	Glu	Gly	Tyr	Gly	Gln	Leu	1	5	10	15
Cys	Ser	Ile	Tyr	Leu	Lys	Leu	Leu	Arg	Thr	Lys	Met	Glu	Tyr	His	20	25	30	
Thr	Lys	Asn	Pro	Arg	Phe	Pro	Gly	Asn	Leu	Gln	Met	Ser	Asp	Arg	35	40	45	
Gln	Leu	Asp	Glu	Ala	Gly	Glu	Ser	Asp	Val	Asn	Asn	Phe	Phe	Gln	50	55	60	
Leu	Thr	Val	Glu	Met	Phe	Asp	Tyr	Leu	Glu	Cys	Glu	Leu	Asn	Leu	65	70	75	
Phe	Gln	Thr	Val	Phe	Asn	Ser	Leu	Asp	Met	Ser	Arg	Ser	Val	Ser	80	85	90	
Val	Thr	Ala	Ala	Gly	Gln	Cys	Arg	Leu	Ala	Pro	Leu	Ile	Gln	Val	95	100	105	
Ile	Leu	Asp	Cys	Ser	His	Leu	Tyr	Asp	Tyr	Thr	Val	Lys	Leu	Leu	110	115	120	
Phe	Lys	Leu	His	Ser	Cys	Leu	Pro	Ala	Asp	Thr	Leu	Gln	Gly	His	125	130	135	
Arg	Asp	Arg	Phe	Met	Glu	Gln	Phe	Thr	Lys	Leu	Lys	Asp	Leu	Phe	140	145	150	
Tyr	Arg	Ser	Ser	Asn	Leu	Gln	Tyr	Phe	Lys	Arg	Leu	Ile	Gln	Ile	155	160	165	
Pro	Gln	Leu	Pro	Glu	Asn	Pro	Pro	Asn	Phe	Leu	Arg	Ala	Ser	Ala	170	175	180	
Leu	Ser	Glu	His	Ile	Ser	Pro	Val	Val	Val	Ile	Pro	Ala	Glu	Ala	185	190	195	
Ser	Ser	Pro	Asp	Ser	Glu	Pro	Val	Leu	Glu	Lys	Asp	Asp	Leu	Met	200	205	210	
Asp	Met	Asp	Ala	Ser	Gln	Gln	Asn	Leu	Phe	Asp	Asn	Lys	Phe	Asp	215	220	225	
Asp	Ile	Phe	Gly	Ser	Ser	Phe	Ser	Ser	Asp	Pro	Phe	Asn	Phe	Asn	230	235	240	
Ser	Gln	Asn	Gly	Val	Asn	Lys	Asp	Glu	Lys	Asp	His	Leu	Ile	Glu	245	250	255	
Arg	Leu	Tyr	Arg	Glu	Ile	Ser	Gly	Leu	Lys	Ala	Gln	Leu	Glu	Asn	260	265	270	

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Met	Lys	Thr	Glu	Ser	Gln	Arg	Val	Val	Leu	Gln	Leu	Lys	Gly	His	275	280	285
Val	Ser	Glu	Leu	Glu	Ala	Asp	Leu	Ala	Glu	Gln	Gln	His	Leu	Arg	290	295	300
Gln	Gln	Ala	Ala	Asp	Asp	Cys	Glu	Phe	Leu	Arg	Ala	Glu	Leu	Asp	305	310	315
Glu	Leu	Arg	Arg	Gln	Arg	Glu	Asp	Thr	Glu	Lys	Ala	Gln	Arg	Ser	320	325	330
Leu	Ser	Glu	Ile	Glu	Arg	Lys	Ala	Gln	Ala	Asn	Glu	Gln	Arg	Tyr	335	340	345
Ser	Lys	Leu	Lys	Glu	Lys	Tyr	Ser	Glu	Leu	Val	Gln	Asn	His	Ala	350	355	360
Asp	Leu	Leu	Arg	Lys	Asn	Ala	Glu	Val	Thr	Lys	Gln	Val	Ser	Met	365	370	375
Ala	Arg	Gln	Ala	Gln	Val	Asp	Leu	Glu	Arg	Glu	Lys	Lys	Glu	Leu	380	385	390
Glu	Asp	Ser	Leu	Glu	Arg	Ile	Ser	Asp	Gln	Gly	Gln	Arg	Lys	Thr	395	400	405
Gln	Glu	Gln	Leu	Glu	Val	Leu	Glu	Ser	Leu	Lys	Gln	Glu	Leu	Gly	410	415	420
Thr	Ser	Gln	Arg	Glu	Leu	Gln	Val	Leu	Gln	Gly	Ser	Leu	Glu	Thr	425	430	435
Ser	Ala	Gln	Ser	Glu	Ala	Asn	Trp	Ala	Ala	Glu	Phe	Ala	Glu	Leu	440	445	450
Glu	Lys	Glu	Arg	Asp	Ser	Leu	Val	Ser	Gly	Ala	Ala	His	Arg	Glu	455	460	465
Glu	Glu	Leu	Ser	Ala	Leu	Arg	Lys	Glu	Leu	Gln	Asp	Thr	Gln	Leu	470	475	480
Lys	Leu	Ala	Ser	Thr	Glu	Glu	Ser	Met	Cys	Gln	Leu	Ala	Lys	Asp	485	490	495
Gln	Arg	Lys	Met	Leu	Leu	Val	Gly	Ser	Arg	Lys	Ala	Ala	Glu	Gln	500	505	510
Val	Ile	Gln	Asp	Ala	Leu	Asn	Gln	Leu	Glu	Glu	Pro	Pro	Leu	Ile	515	520	525
Ser	Cys	Ala	Gly	Ser	Ala	Asp	His	Leu	Leu	Ser	Thr	Val	Thr	Ser	530	535	540

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Ile Ser Ser Cys	Ile Glu Gln Leu Glu	Lys Ser Trp Ser Gln Tyr	545	550	555
Leu Ala Cys Pro	Glu Asp Ile Ser Gly	Leu Leu His Ser Ile Thr	560	565	570
Leu Leu Ala His	Leu Thr Ser Asp Ala	Ile Ala His Gly Ala Thr	575	580	585
Thr Cys Leu Arg	Ala Pro Pro Glu Pro	Ala Asp Ser Leu Thr Glu	590	595	600
Ala Cys Lys Gln	Tyr Gly Arg Glu Thr	Leu Ala Tyr Leu Ala Ser	605	610	615
Leu Glu Glu Glu	Gly Ser Leu Glu Asn	Ala Asp Ser Thr Ala Met	620	625	630
Arg Asn Cys Leu	Ser Lys Ile Lys Ala	Ile Gly Glu Glu Leu Leu	635	640	645
Pro Arg Gly Leu	Asp Ile Lys Gln Glu	Glu Leu Gly Asp Leu Val	650	655	660
Asp Lys Glu Met	Ala Ala Thr Ser Ala	Ala Ile Glu Thr Cys Thr	665	670	675
Ala Arg Ile Glu	Glu Met Leu Ser Lys	Ser Arg Ala Gly Asp Thr	680	685	690
Gly Val Lys Leu	Glu Val Asn Glu Arg	Ile Leu Arg Cys Cys Thr	695	700	705
Ser Leu Met Gln	Ala Ile Gln Val Leu	Ile Val Ala Ser Lys Asp	710	715	720
Leu Gln Arg Glu	Ile Val Glu Ser Gly	Arg Gly Thr Ala Ser Pro	725	730	735
Lys Glu Phe Tyr	Ala Lys Asn Ser Arg	Trp Thr Glu Gly Leu Ile	740	745	750
Ser Ala Ser Lys	Ala Val Gly Trp Gly	Ala Thr Val Met Val Asp	765	770	775
Ala Ala Asp Leu	Val Val Gln Gly Arg	Gly Lys Phe Glu Glu Leu	780	785	790
Met Val Cys Ser	His Glu Ile Ala Ala	Ser Thr Ala Gln Leu Val	795	800	805
Ala Ala Ser Lys	Val Lys Ala Asp Lys	Asp Ser Pro Asn Leu Ala	810	815	820

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Gln	Leu	Gln	Gln	Ala	Ser	Arg	Gly	Val	Asn	Gln	Ala	Thr	Ala	Gly
				825					830					835
Val	Val	Ala	Ser	Thr	Ile	Ser	Gly	Lys	Ser	Gln	Ile	Glu	Glu	Thr
				840					845					850
Asp	Asn	Met	Asp	Phe	Ser	Ser	Met	Thr	Leu	Thr	Gln	Ile	Lys	Arg
				855					860					865
Gln	Glu	Met	Asp	Ser	Gln	Val	Arg	Val	Leu	Glu	Leu	Glu	Asn	Glu
				870					875					880
Leu	Gln	Lys	Glu	Arg	Gln	Lys	Leu	Gly	Glu	Leu	Arg	Lys	Lys	His
				885					890					895
Tyr	Glu	Leu	Ala	Gly	Val	Ala	Glu	Gly	Trp	Glu	Glu	Gly	Thr	Glu
				900					905					910
Ala	Ser	Pro	Pro	Thr	Leu	Gln	Glu	Val	Val	Thr	Glu	Lys	Glu	
				915					920				924	

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CLAIMS

1. A cDNA molecule comprising the sequence given by Seq. ID No. 1.
2. A cDNA molecule comprising the sequence given by Seq. ID No. 5.
3. A polypeptide comprising the sequence given by Seq. ID. No. 2.
4. A polypeptide comprising the sequence given by Seq. ID. No. 6.
5. A chimeric gene or plasmid comprising at least nucleotides 314 to 1955 of the Huntington's Disease gene and an activating or DNA binding domain suitable for use in a yeast multi-hybrid assay.
6. The chimeric gene or plasmid according to claim 5, wherein the Huntington's Disease gene encodes a polyglutamine tract having a length of 35 or fewer residues.
7. The chimeric gene or plasmid according to claim 5, wherein the Huntington's Disease gene encodes a polyglutamine tract having a length of 36 or more residues.
8. A method for ameliorating the effects of Huntington's disease in a patient expressing Huntingtin protein with an expanded CAG repeat region, comprising the step of increasing the amount of an expressed HD-interacting polypeptide in the brain of the patient, wherein the expressed HD-interacting polypeptide interacts less well with expanded Huntingtin than with Huntingtin having a CAG repeat region containing 15 to 35 repeats and facilitates the incorporation of Huntingtin into brain cell membranes.
9. The method according to claim 8, wherein the expressed HD-interacting polypeptide comprises the sequence given by Seq. ID No. 2.

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1 10. An antibody which binds to a polypeptide having the sequence given by
2 Seq. ID. No. 2.

1 11. The antibody of claim 10, wherein the antibody binds to amino acids
2 76-91 of the polypeptide having the sequence shown in Seq. ID No. 2.

1 12. An expression vector for expression of a gene in a mammalian host
2 comprising a region encoding an HD-interacting polypeptide, wherein the HD-interacting
3 polypeptide interacts less well with expanded Huntingtin than with Huntingtin having a CAG
4 repeat region containing 15 to 35 repeats and facilitates the incorporation of Huntingtin into
5 brain cell membranes.

1 13. An expression vector for expression of a gene in a mammalian host
2 comprising a region that is the same as or complementary to Seq. ID NO. 1.

1 14. An expression vector for expression of a gene in a mammalian host
2 comprising a region that is the same as or complementary to Seq. ID NO. 5.

1 15. The expression vector according to claims of claims 12-14, further
2 comprising a region encoding Huntingtin having a polyglutamine tract of 35 or fewer.

1 16. An oligonucleotide probe having a length of from 15-40 bases which
2 specifically and selectively hybridizes with the cDNA given by Seq. ID No. 1 or a sequence
3 complementary thereto.

1/1

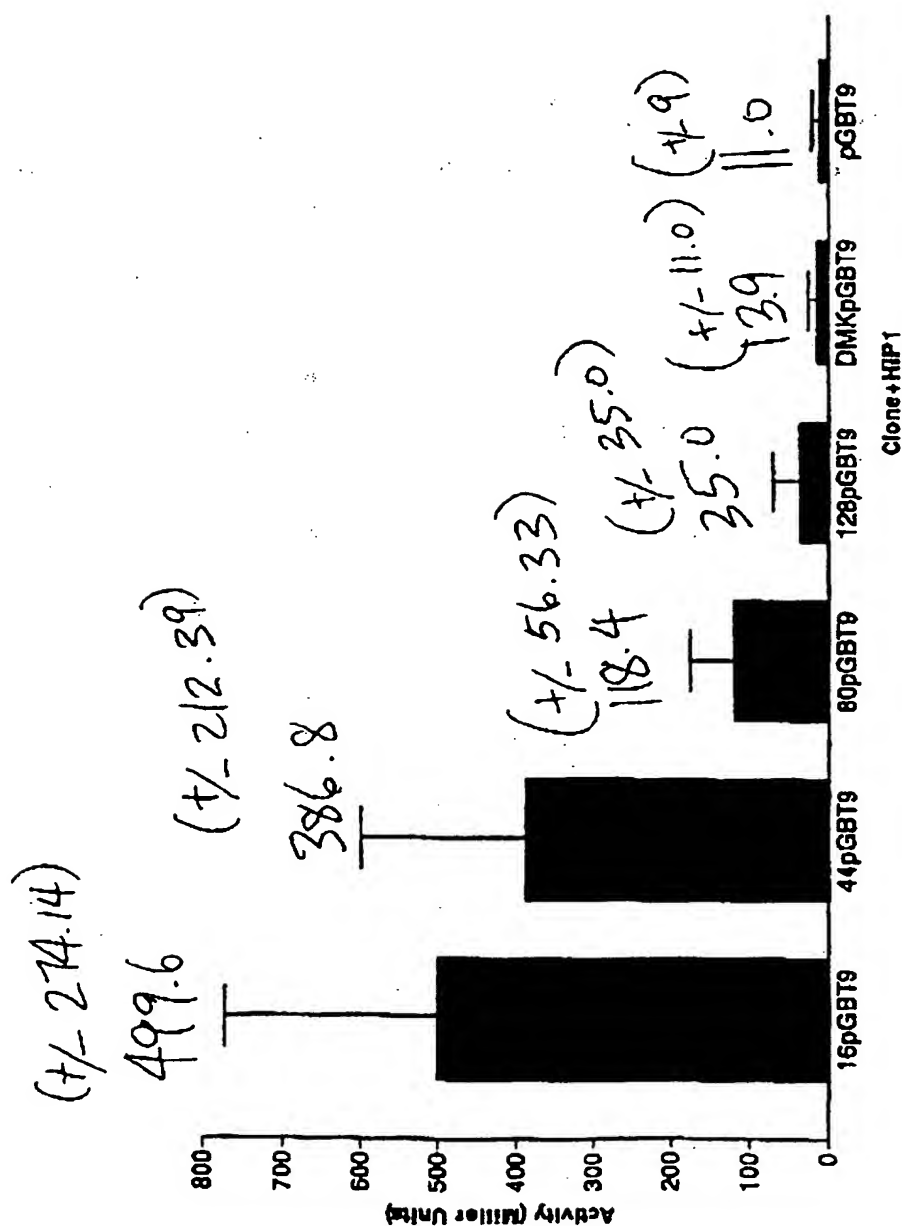


Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18370

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.
US CL : Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 6, 69.1, 172.3; 514/44, 2; 935/62, 52, 56, 65, 34; 536/24.5, 23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, BIOSIS, MEDLINE, EMBASE, CAPLUS, WPIDS, APS, INPADOC
search terms: interacting protein, huntingtin, huntington, cag repeat, hip, gene therapy

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0617 125 A2 (THE GENERAL HOSPITAL CORPORATION) 28 September 1994, entire document, especially pages 4-17.	8, 10-12, 16
Y	WO 94/24279 (BERGMANN ET AL.) 27 October 1994, entire document, especially pages 13 and 28-39.	8, 12 and 16
Y	EP 0 614 977 A2 (THE GENERAL HOSPITAL CORPORATION) 14 September 1994, entire document.	1-16
Y	BIAOYANG et al. Sequence of the Murine Huntington Disease Gene: Evidence for Conservation, and polymorphism in a triplet (CCG) Repeat Alternate Splicing. Human Molecular Genetics. January 1994, Vol. 3, No. 1, pages 85-92, see entire document.	8-9, 12-15

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

15 JANUARY 1997

Date of mailing of the international search report

14 MAR 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer
KAREN M. HAUDA

gab for

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/18370

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	GOLDBERG et al. Cleavage of Huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. Nature Genetics. 13 August 1996, Vol. 13, No. 4, pages 442-449, see entire document.	6, 7
X,P	KALCHMAN et al. HIP-2 - A Huntingtin interacting protein: Insight into the Catabolism of the HD gene product. American Journal of Human Genetics. 02 November 1996, Vol. 59, Supplement 4, page A152, see entire document.	1-4, 12-14, and 16
Y,P		5-11 and 15

INTERNATIONAL SEARCH REPORT

International application No. .
PCT/US96/18370

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-4, 9-11 and 13-16 (in part)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claims recite sequence ID numbers, but no sequence disk was submitted. Due to the length of the sequences, a search could not properly be completed on the sequence ID numbers claimed.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/18370

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 38/00, 15/31, 15/09, 48/00; C12N 15/79, 15/63, 15/00; C07K 16/00; C07H 21/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/320.1, 6, 69.1, 172.3; 514/44, 2; 935/62, 52, 56, 65, 34; 536/24.5, 23.1